Immunology 1986 **58** 157–160

Production of human monoclonal IgG and IgM antibodies with anti-D (rhesus) specificity using heterohybridomas

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Accepted for publication 18 December 1985

SUMMARY

Heterohybridomas secreting human IgM and IgG anti-D antibodies of the rhesus blood group system have been established by fusion of EBV-transformed anti-D secreting cells with the mouse myeloma cells X63-Ag8.653. Both classes of antibody reacted with all Rh-positive cells, some D^u cells but not with Rh-negative or D^B cells. Concentrations of both antibodies reached between 25 μg/ml and 50 μg/ml in the culture supernatants. The cell lines have been maintained in culture for 14 months and have been shown to be suitable for large-scale production of antibody.

INTRODUCTION

The human anti-D antibody of the Rh system is widely used, both for blood typing in transfusion practice and prophylactally in the prevention of haemolytic disease of the newborn (Mollison, 1983). At the present time, antibodies are obtained from donors immunized during pregnancy or following a mismatched blood transfusion, or by the deliberate immunization of volunteers. In order to obtain adequate plasma anti-D concentrations many donors require boosting injections of red cells, and this procedure carries the risks common to receiving any transfusion of red cells. Moreover, the agglutinating IgM anti-D, which is particularly valuable in Rh typing, is very difficult to obtain as it is rarely produced in high titre by immunized donors. It would thus be of great advantage if anti-D could be produced in tissue culture. It has previously been shown (Boylston *et al.*, 1980; Melamed *et al.*, 1985) that B lymphocytes from anti-D donors can produce anti-D in tissue culture after infection with Epstein–Barr virus (EBV), but unfortunately these lymphoblastoid cells usually cease production of anti-D after a few months. We now report that the lymphoblastoid cell lines can be stabilized by the formation of a heterohybridoma following fusion with the mouse myeloma X63-Ag8.653. The production of both an IgM and an IgG anti-D has continued over a 14-month observation period.

MATERIALS AND METHODS

Haemagglutination and quantification of antibody

Detection and assay of the IgM and IgG anti-D obtained from tissue culture wells were carried out by agglutination in microtitre plates using native and papain-treated red cells, respectively, at a final concentration of 0·5% cells. The sensitivity of the assay for both antibody classes is approximately 1 ng/ml. The potency of agglutination of the monoclonal MAD-2 was compared to the US Office of Biologics, Research and Review IgM anti-D standard preparation, Lot 9, and carried out according to the suppliers instructions. The serological characteristics of the MAD-2 and GAD-2 monoclonals were investigated by standard blood transfusion techniques. Quantitation of the anti-D present in culture supernatants was carried out by an inhibition immunoassay. ^{125}I-labelled anti-D and R_2R_2 red cells were incubated in the presence and absence of culture supernatant and the reaction mixture brought into equilibrium. The amount of ^{125}I-labelled anti-D bound to the red cells was then determined, and the concentration of anti-D in the culture supernatant was calculated by comparison with a standardization curve using the anti-D International Standard (68/419) (N. C. Hughes-Jones and B. D. Gorick, in preparation).

Donor of B lymphocytes

The buffy coat was obtained from an Rh-negative donor who had been initially immunized with six injections of Rh-positive cells (R_2R_2) at the Cambridge Blood Transfusion Centre 10 years previously. The most recent boosting injection was 7 weeks prior to bleeding. Only IgG anti-D was present at the time of bleeding, but IgM antibody had been present 1 week after boosting.

Establishment of lymphoblastoid cells (LCL)

The B lymphocytes were separated from red cells, platelets and T cells using Ficoll–Hypaque, Percoll and sheep red cells, and then exposed to Epstein–Barr virus (EBV) as previously described (Melamed *et al.*, 1985).

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Fusion

The mouse myeloma X63-Ag8.653 was grown in RPMI-1640 supplemented with 10% fetal calf serum, 2 mM glutamine, in an atmosphere of 5% CO_2 in air, at 37°. Under these conditions, the cell line had a doubling time of 18–20 hr, and was maintained in log phase growth by daily subculturing. Prior to fusions, the viability of the cells (determined by nigrosine dye exclusion) exceeded 95%. Periodically, 8-azaguanine was included in the culture medium at 20 μg/ml to eliminate variants that would survive in hypoxanthine-aminopterin-thymidine (HAT) medium.

LCL (1×10^7) and myeloma cells (1×10^7) were added together in a 25 ml plastic container, diluted with phosphate-buffered saline (NaCl 150 mM; PO_4 10 mM) and spun to a common pellet for 10 min at 200 *g*. After loosening the pellet, 1 ml of 45% polyethylene glycol 4000 (Merck, Darmstadt, FRG) and 5% dimethylsulphoxide in phosphate-buffered saline was added dropwise over a period of 1 min with constant agitation. Agitation was continued in a water bath at 37° for 90 seconds, after which the cells were slowly diluted with saline at room temperature. The cells were centrifuged for 5 min at 200 *g*, then gently resuspended in complete RPMI-1640, and distributed in 96-well trays preseeded with mouse peritoneal cells (2×10^4 per well), such that each well received $1 \times \times 10^5$ myeloma cells in 100 μl of medium. After 24 hr incubation, 100 μl of medium containing twice-concentrated HAT (Littlefield, 1964) and 2 μM ouabain was added. HAT prevents the outgrowth of unfused mouse myeloma cells and oubain kills unfused LCL.

Wells were examined after 7 days when feeding was started by replacing half the medium with fresh medium containing HAT and 1 μM ouabain. Feeding was repeated every 2 or 3 days. Wells positive for growth were screened for immunoglobulin or antibody production when cells covered half the bottom of the well. Hybrids of interest were cloned directly from the 96-well trays. HAT/ouabain selection was maintained for 3 weeks after fusion, and the cells passaged through HT-containing medium before culturing in standard tissue culture medium.

Cloning of hybrids

Hybrids were transferred singly from a culture in log phase growth by use of a micromanipulator and microscope to individual wells of a 96-well tray preseeded with mouse peritoneal feeder cells. In order to ensure that only single cells were transferred, cells were only picked up when alone in the field of a high power lens, and expelled into an intermediate vessel for examination before being moved to the final well.

Assay for human immunoglobulin and chromosome analysis

Supernatants were screened for the production of human immunoglobulin by an enzyme-linked immunoassay (K. Thompson, D. W. Hough and P. J. Maddison, submitted). EIA trays (Dynatech, Billingshurst, Sussex) were coated with goat anti-polyvalent human immunoglobulin antibodies (Sigma, Poole, Dorset). Culture supernatants were then incubated in the coated trays. Trays were washed with phosphate-buffered saline containing 0·1% Tween 20, and then incubated with anti-human heavy chain or light chain specific antibodies conjugated to alkaline phosphatase (Sigma). After a final wash, trays were incubated with *p*-nitrophenyl phosphate and colour changes monitored on a 'Titertek Multiskan' EIA reader. The IgG subclass was determined by agglutination of red cells using class-specific anti-sera (Dutch Red Cross, Amsterdam, The Netherlands). Chromosome preparations were made by standard procedures and G-banded (Freshney, 1983).

RESULTS

Establishment of the lymphoblastoid cell line

The EBV-transformed B cells from the donor were grown in 192 flat-bottomed wells (200 μl) at an initial density of 5×10^5/ml. Growth was established in all wells and anti-D was present in 71% of wells 2 weeks later. Two of the wells were selected for their vigorous growth and high anti-D titres (> 2000) and grown on to reach 80-cm^2 flasks. One of the wells was producing an IgG anti-D, and the other produced an IgM anti-D. Two months later, at the time of fusion, the IgG anti-D production rate was declining (titre 81) but production of the IgM anti-D was stable (titre approximately 5000). The supernatants of both cultures contained α, γ and μ heavy chains and kappa and lambda light chains.

Formation of heterohybridomas and cloning

All wells from the two fusions showed vigorous growth. No growth was seen in any well from two mock fusions in which the polyethylene glycol step was omitted. Four weeks after fusion, 49 out of 96 wells from the IgG anti-D fusion and 30 out of 96 wells from the IgM anti-D fusion were producing anti-D titres greater than 100. One well from each fusion was selected for good growth and high anti-D titre, and cloned by micromanipulation. The cloned cells were recloned after 4 weeks. After the first cloning, all the subclones (27/27) of the IgG-producing line and 10 out of 13 of the IgM-producing line were positive for anti-D. After the second cloning, all of the subclones (41/41) of the IgG line, and 33 out of 40 subclones of the IgM line, were positive for anti-D production. Both monoclonals have lambda light chains and the IgG belongs to the IgG_3 subclass.

Hybridization efficiency

In an initial experiment designed to determine the suitability of X63-Ag8.653 as a fusion partner, the fusion efficiency was found to be approximately 1 in 1×10^4.

Large scale production

Two wells were selected, one for high IgG (GAD-2) and the other for high IgM (MAD-2) anti-D production, and the volume of the cultures expanded; growth established in either 2-litre roller cultures or 20-litre spinner cultures. The percentage of cells producing IgM anti-D when the cells were first put into roller cultures was 90% as determined by recloning, and this level was unchanged 3 months later. After 5 months in culture, the IgG-producing cells showed a retardation in the rate of growth and a fall in anti-D production to approximately 10% of its peak level. This change may have been due to a mycoplasma infection prevalent in the laboratory at that time. Cloning showed that only 10% of the cells were producing anti-D and the line was re-established from one of these clones with anti-D production returning to its original level (titre 2000–6000). The cultures are continuing to produce anti-D 14 months after the

time of fusion. Haemagglutination titres of supernatants from both hybrids vary between 2000 and 6000.

The production rate of the IgG antibody was about 50 μg/10^6 cells/24 hr (approximately 2000 molecules/second/cell) and concentrations in the supernatant varied between 25 μg and 50 μg anti-D/ml. IgM anti-D concentrations after 24 hr of cultures at 1×10^6 cells/ml were also 25–50 μg/ml.

Functional studies on the antibodies

The IgM anti-D agglutinated all D-positive red cells tested and most D^u, cells but not with Rh-negative or D^B cells. Details are given in Table 1. When added at a final concentration of 5 μg/ml to a 1% suspension of D-positive cells, it brought about complete agglutination in about 30 seconds. In a potency test comparing the IgM anti-D (MAD-2) with the US standard, MAD-2 gave a titre of 4000 compared to a value of 128 given by the US standard. The antibody has been found to be stable at 4° over an 8-month period.

The IgG anti-D reacted with all D-positive red cells, one out of five D^u samples, but not with Rh-negative or D^B cells. Preliminary experiments by Dr E. Wiener, St Mary's Hospital Medical School, London, (unpublished observations) have shown that the IgG3 monoclonal opsonizes red cells for phagocytosis.

Chromosomes

Karyotype analysis was carried out on the cells derived from the IgG-producing clone to demonstrate their hybrid character. All cells contained both the metacentric (human) and acrocentric (mouse) chromosomes. The number of chromosomes in 29 cells were determined. Twenty of the cells had between 80 and 120 chromosomes, the modal value being about 100. Three had fewer than 80 and six fell in the range 120–200. Human chromosome 22, on which the lambda gene resides, was identified in several of the cells. The X63-Ag8.653 cell has a model number of 49 chromosomes (K. Thompson, D. W. Hough and P. J. Maddison, submitted).

Table 1. The results of agglutination tests using red cells of differing genotypes

Cell genotype	Number of donors	IgM (MAD-2)	IgG (GAD-2)
OR_1r		+	+
OR_2r		+	+
OR_oR		+	+
Or'r		–	–
Or''r		–	–
A_1rr		–	–
Orr		–	–
D^u	5	4/5*	1/5†
		5/5†	
D^B	3	0/3	0/3
IgG-coated‡		–	Not tested
C3–C4-coated‡		–	Not tested

+, Agglutination present.
–, No agglutination.
* Four out of five positive after 1 min incubation.
† After 30 min incubation.
‡ Rh-negative cells strongly coated with IgG anti-c or Rh-negative cells coated with the complement components C3 and C4. These cells were included as controls to show that the IgM anti-D did not cause non-specific agglutination of cells coated with 'non-anti-D' IgG.

DISCUSSION

It has been a common, although infrequently reported observation that human B lymphocytes infected with Epstein–Barr virus are not stable, and that cell lines producing a specific antibody usually cease production after a few months (Melamed *et al.*, 1985). In this report, we show that it is possible to give stability to the lymphoblastoid lines by fusing them with the mouse myeloma X63-Ag8.653. Karyotype analysis showed that the resultant cell lines contained both human and mouse chromosomes. The first report of a successful fusion between a mouse myeloma line (TEPC-15) and human peripheral blood B lymphocytes was that of Schwaber & Cohen (1973). The resultant heterohybridoma secreted both mouse and human Ig. Further reports used B cells from patient's spleens (Nowinski *et al.*, 1980) lymph nodes (Schlom, Wunderlich & Teramoto, 1980) or peripheral blood (Croce *et al.*, 1979; Lane *et al.*, 1982). The mouse myeloma lines NSI or SP1 were used in these fusions. Kozbor *et al.* (1982) used EBV-infected peripheral blood B cells and the same mouse myeloma that we used here (X63-Ag8.653) and the resultant hybridoma secreted an IgG anti-tetanus antibody for over 9 months. Bron *et al.* (1984) have reported the establishment of a heterohybridoma producing anti-D where the myeloma fusion partner was itself a heterohybridoma formed between a human myeloma cell and the mouse myeloma X63-Ag8.653, and Foung *et al.* (1984) have reported the production of an anti-A by a heterohybridoma formed originally from the mouse line SP2/08AZ.

The use of fusion partners resulting in the highest yield of Ig-secreting hybrids is important as the number of peripheral blood cells potentially secreting anti-D is only 1 in 10^3 or 10^4 of the total B-cell population in the donor's peripheral blood (Elson & Bradley, 1970). The mouse myeloma X63-Ag8.653 has been found to be an efficient fusion partner with human peripheral blood lymphocytes, compared to other human or mouse cell lines (K. Thompson, D. W. Hough and P. J. Maddison, submitted). It also has the advantage of producing no mouse immunoglobulin of its own, and thus hybrids derived from it produced only the desired antibodies. There is considerable advantage in transforming the donor's B cells with EBV prior to fusion since this increases the fusion efficiency (about 1 in 10^4) and allows selection and expansion of cells secreting the desired antibodies.

Heterohybridomas have a reputation for chromosomal instability, and use is made of this fact for designating genes to chromosomes (Ruddle, 1973). However, loss of chromosomes usually occurs within a few weeks of fusion, and cells that survive for several months would appear to have a good chance of remaining stable. As with all hybrids, some loss of Ig production may be expected from time to time, but the line can readily be re-established by recloning from an anti-D-secreting cell.

The IgM anti-D appears to be a very suitable antibody for

use in Rh typing of blood transfusion recipients. The undiluted culture supernatants were found to be about 32 times more potent than the reference standard, and can be thus diluted for routine use. The IgM anti-D does not react with the rare D^B cells. This does not matter for recipients who would be classed as Rh-negative and thus receive Rh-negative blood. Donors classed as Rh-negative with this IgM anti-D would have to be screened with a polyclonal IgG anti-D in order to determine the presence of the D^u or D^B classes.

The IgG3 anti-D appears to be capable of bringing about opsonization of Rh-positive cells when tested using human macrophages *in vitro*. It thus has the potential for destroying Rh-positive red cells present *in vivo* in Rh-negative people, and therefore it may be possible for this monoclonal anti-D to replace polyclonal anti-D in the prophylaxis of haemolytic disease of the newborn.

Both the IgG- and IgM-producing heterohybridomas have been found to grow well in large-scale bulk culture and to produce sufficient antibody for intended use. A production rate of approximately 2–3 litres/week provides sufficient IgM anti-D for blood typing in a population of 50 million people with the existing transfusion practices, and approximately 20 litres/week will supply sufficient anti-D for passive immunization.

ACKNOWLEDGMENTS

We would like to thank Dr Marcella Contreras for subtyping the IgG anti-D, Dr Edith Wiener for the studies on phagocytosis, and Dr Ann Dain for chromosome counting.

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